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Taurine in the mammalian cerebellum: Demonstration by autoradiography with [^{3}H]taurine and immunocytochemistry with antibodies against the taurine-synthesizing enzyme, cysteine-sulfinic acid decarboxylase

(Purkinje cell/transmitters/cerebellar nuclei)

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ABSTRACT Taurine neurons and their dendrites and axons were visualized in the mammalian cerebellum by autoradiography, after *in vivo* injections of [^{3}H]taurine directly into the cerebellar cortex or deep cerebellar nuclei, and by immunocytochemistry at the light- and electron-microscope levels with antibodies against cysteine-sulfinic acid decarboxylase (CSADCase; L-cysteine-sulfinate carboxylyase, EC 4.1.1.29). Uptake and sequestration of [^{3}H]taurine labeled numerous Purkinje cell somata, primary dendrites, and axons; many granule cell somata, dendrites, and parallel fibers; stellate, basket, and Golgi cells; the larger neurons in all deep cerebellar nuclei; the largest neurons in the lateral vestibular nucleus; and, more rarely, Purkinje cell axonal terminals in the neuropil. The label at all sites was diminished by preinjection into the cerebellum of hypotaurine, *p*-chloromercuriphenylsulfonic acid, or β-alanine, and was virtually eliminated by strychnine. Immunocytochemical labeling with polyclonal antibodies directed against CSADCase, the enzyme responsible for the synthesis of hypotaurine from cysteine sulfinic acid and taurine from cysteic acid, had a similar distribution. In electron micrographs, immunoreactivity within Purkinje cell somata and dendrites was localized to the Golgi apparatus, the inner plasma membrane, and condensed nonmembranous foci (120 nm in diameter) marked by clumps of peroxidase reaction product. Large Nissl bodies were usually not CSADCase immunoreactive. Numerous immunoreactive granule cells, dendrites, and parallel fibers were recognized. Pretreatment of the animals with colchicine increased the intensity of CSADCase immunoreactivity but did not change the number or distribution of labeled cells. These experiments indicate that taurine is synthesized and involved in a specific uptake process by cerebellar neurons. Neuroglial cells do not synthesize taurine but some neuroglia take up [^{3}H]taurine. These findings call for a reexamination of the physiological function of taurine in the cerebellum. A hypothesis is proposed that taurine may be involved in the regulation of calcium, in dendritic spike generation, and in the inhibition of impulse propagation in major Purkinje cell dendrites.

Taurine (2-aminoethanesulfonic acid) has a simple structure, is chemically stable, and is the most abundant free amino acid in animals. Experiments (1, 2) have suggested a possible neurotransmitter role; data from microiontophoretic studies on brain stem neurons (3) and from studies on uptake and release of taurine in the brain *in vivo* (4), in brain or spinal cord slices (5, 6, 7), in synaptosomes (8, 9), in the retina (10), and in cerebellar stellate cells (11) appear to support this hypothesis. In mammalian nervous tissues, taurine has a number of biosynthetic routes; the major two involve the transsulfuration pathway of methionine to cysteine, the subsequent formation of cysteic acid or cysteine sulfinic acid, and their conversion to taurine. The decarboxylation of either cysteine sulfinic acid or cysteic acid is believed to be catalyzed by a single protein, cysteine-sulfinic acid decarboxylase (CSADCase; L-cysteine-sulfinate carboxylyase, EC 4.1.1.29) or cysteic acid decarboxylase. The activity of CSADCase in 1-yr-old calf brain has been found to be highest in cerebellar cortex and in a descending order of intensity in the mesencephalon, diencephalon, corpus striatum, cerebral cortex, and white matter (12). CSADCase has been purified recently from bovine brain, and polyclonal antibodies have been raised and characterized against it (unpublished data). In the present study, the sites of synthesis, uptake, and transport of taurine were localized in cerebellum by [^{3}H]taurine autoradiography and immunocytochemistry at the light microscope and ultrastructural levels with anti-CSADCase antibodies.

MATERIALS AND METHODS

Autoradiography. [^{3}H]Taurine (New England Nuclear; specific activity, 20–40 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was injected directly through glass micropipettes (tip diameter, 10 μm) into the cerebellar cortex and deep cerebellar nuclei of anesthetized rats (Charles River Breeding Laboratories; 200-g body weight). [^{3}H]Taurine was injected into four groups of animals, each group receiving a different concentration: 50 μM, 5 μM, 0.5 μM, and 50 nM. The animals received several injections, each <1.0 μl, for a total of 200 μCi in animals receiving the highest concentration to 0.2 μCi in those receiving the lowest concentration. Ten minutes after the last injection, the animals were perfused through the heart with 2% glutaraldehyde/0.12 M phosphate buffer, pH 7.4 (13, 14). The cerebellum was fixed overnight and then soaked in 0.1 M Tris, pH 7.4/5% sucrose. Serial frozen sections (10 μm thick) were obtained on a cryostat (Dittes), and autoradiographs were prepared with NTB-2 emulsion (Kodak) (14, 15) by the dipping method. Autoradiographs were exposed for 4 wk and developed in D19 (Kodak). In control experiments, each of four additional groups of rats were pretreated with one of the following substances: hypotaurine (1 mM), *p*-chloromercuriphenylsulfonic acid (0.1 mM), β-alanine (10 mM), or strychnine (1 mM). In each animal, the agent was delivered by glass micropipettes in three penetrations into the cerebellum for a total of 2 μl over 2–5 min. [^{3}H]Taurine was injected into the same sites in the cerebellum. The animal was perfused 10 min later through the heart with fixatives, and the tissues were processed as described.

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Abbreviations: CSADCase, cysteine-sulfinic acid decarboxylase; PAP, peroxidase-antiperoxidase.

Immunocytochemistry. The indirect peroxidase-labeled second antibody method, the peroxidase-antiperoxidase (PAP) method, and normal rats (200 g; Charles River Labs) were used in these studies (16, 17). In addition, rats were injected intraperitoneally with demecolcine (1 mg/kg of body weight) 16 hr before fixation to retard axoplasmic transport. Each was fixed after anesthesia by perfusion through the heart with fixative. The fixatives consisted of 4% HCHO/0.12 M phosphate buffer beginning at pH 6.5 and ending at pH 11 or 4% HCHO/0.04 M phosphate buffer, pH 7.4, with sodium periodate (0.55 g/liter) and lysine (3.42 g/liter). For electron-microscope studies, 0.1% glutaraldehyde was added to the fixative. Tissues were stored overnight in fixative with or without sucrose (8.5%) and subsequently were sectioned at 50 μm on a Vibratome. For staining, loose sections were incubated in individual chambers in plastic trays in the following sequence: in 25 mM hydroxylamine (15–60 min, to block unreacted aldehyde groups from the fixation); in 10% normal goat serum with 0.02% Triton X-100 or 0.1% saponin for 1 hr to block nonspecific immunoglobulin binding (thus reducing background) and to aid penetration by antibodies; buffer rinses; anti-CSADCase antibodies at dilutions of 1:40 to 1:2,000 for 16 hr at 4°C or 1–2 hr at room temperature or 37°C; buffer rinses; goat anti-rabbit antiserum (1:100 dilution) or peroxidase-conjugated goat anti-rabbit antiserum (1:100 dilution); buffer rinses; PAP complex (1:100 dilution for 90 min at 37°C); buffer rinses; and finally diaminobenzidine tetrahydrochloride with H_2O_2. For electron microscopy, no detergents were used and individual sections were postfixed in 1% glutaraldehyde/1% HCHO/0.12 M phosphate buffer with osmium tetroxide, dehydrated in methanol, and embedded in Spurr's low viscosity resin.

The anti-CSADCase antibodies are polyclonal, raised in rabbits against CSADCase, and purified to homogeneity from bovine brain. The purity of the protein was established by polyacrylamide gel electrophoresis and immunodiffusion testing (18). Antibodies to CSADCase appear specific to this antigen as established by double immunodiffusion, immunoelectrophoresis, microcomplement-fixation, and enzyme-inhibition tests (unpublished data). No crossreactivity exists between CSADCase and glutamic acid decarboxylase. Controls were done with serum from nonimmunized rabbits, serum from rabbits immunized against ovalbumin, and immune serum preabsorbed with CSADCase.

RESULTS

Cellular locations of [^{3}H]taurine uptake were visualized in all autoradiograms produced at the four [^{3}H]taurine concentrations administered. In the cerebellar cortex, the most conspicuous label was found over many (but not all) Purkinje cells, particularly over the main dendritic stems and major bifurcations, less

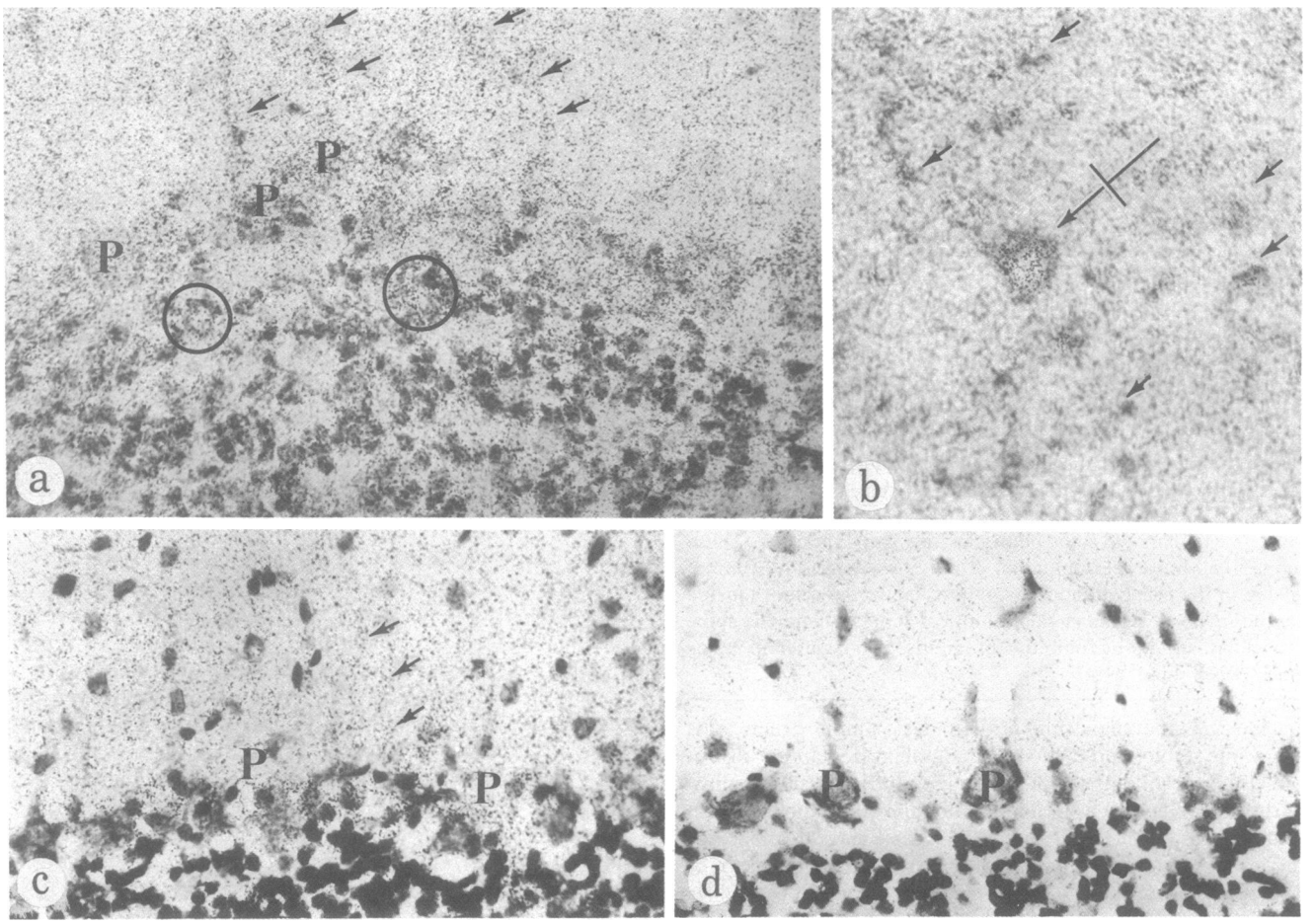

FIG. 1. Rat cerebellum labeled by autoradiography after *in vivo* microinjections of [^{3}H]taurine. (*a*) Cerebellar cortex in normal rat with labeling in Purkinje cell somata (P), major dendrites (arrows), and in the granular layer, particularly granule cells (encircled). (*b*) Dentate nucleus in normal rat with labeled larger nuclear cell (crossed arrow) and processes in the neuropil (small arrows). (*c*) Cerebellar cortex after pretreatment with *p*-chloromercuriphenylsulfonic acid, an uptake inhibitor, by *in vivo* injection 10 min prior to injection of [^{3}H]taurine. There is diminished labeling of Purkinje cells (P) and dendrites (arrows). (*d*) Pretreatment with strychnine by *in vivo* injection, followed by injection of [^{3}H]taurine. Only a background level of grains appears. Purkinje cells (P) are indicated. All preparations were counterstained with cresyl violet. (×200.)

over the somata, and rarely over the axonal plexus in the deep cerebellar nuclei. About one-third of the stellate, basket, and Golgi cells were also labeled. Granule cells were labeled, and the lower molecular layer had a heavy deposition of label, suggesting contribution by their axons (Fig. 1*a*). In the deep cerebellar nuclei, many of the larger cells were labeled, as were some axons in the neuropil (Fig. 1*b*). Local pretreatment of the injection site with hypotaurine, *p*-chloromercuriphenylsulfonic acid, and β-alanine reduced the intensity of labeling after [^{3}H]taurine administration (Fig. 1*c*). However, virtual elimination of labeling with [^{3}H]taurine was obtained only with strychnine local pretreatment (Fig. 1*d*). [^{3}H]Taurine labeled neuroglial cells, but they formed only a minor proportion of all labeled elements in the cerebellar cortex or nuclei.

In the normal cerebellum CSADCase immunoreactivity was present in most but not all Purkinje neurons, particularly in primary dendritic segments and bifurcations and, to a lesser extent, in the cell somata (Fig. 2*a*). Purkinje axons and terminals were stained markedly less frequently both in the cortex and in the deep nuclei. A number of stellate, basket, and Golgi cells were CSADCase positive in somata, axons, and dendrites. Most remarkably, CSADCase reactivity appeared in many granule cell somata and dendrites in the granular layer and in their axons, the parallel fibers in the molecular layer (Fig. 2 *a* and *c*). In some areas of the cortex, such as in lobules I and X (including the flocculus), most of these various cell types were heavily reactive. In the other lobules, fewer were labeled. In all of the deep cerebellar nuclei, a considerable number of larger neurons (diameters, ≈25–30 μm) were CSADCase positive. Some of these neurons displayed a few CSADCase-positive terminals on their somata, presumably contributions from Purkinje cells in the overlying cortex; however, the stained axons or boutons in the cerebellar nuclei were not numerous (Fig. 2*d*). In colchicine-treated animals, the intensity of CSADCase immunoreactivity was increased, particularly over cell somata and primary dendrites of Purkinje cells, granule cells, and deep nuclear cells (Fig. 2*b*). No stained neuroglial cells were seen.

Electron microscopy confirmed these findings. Representatives of all the neuronal types described above showed CSADCase immunoreactivity (see Fig. 3 *a–e*). The nuclei of all labeled neurons were devoid of reaction product. In Purkinje cell somata (Fig. 3*a*), CSADCase reactivity was concentrated on the inner plasma membrane and in the cytoplasmic matrix around the Golgi apparatus, ribosomes, filaments, and smooth endoplasmic reticulum, generally avoiding organized Nissl substance and mitochondria. In the dendrites, CSADCase reactivity was found around microtubules, smooth endoplasmic reticulum, ribosomes, and filaments, and throughout the cytoplasmic matrix. In the somata and dendrites, CSADCase reactivity appeared to be condensed in 120-nm nonmembranous foci marked by electron-dense peroxidase reaction product. CSADCase immunoreactive postsynaptic elements (Fig. 3*c*) and presynaptic and axonal elements (Fig. 3*e*) were also observed. In axonal varicosities, CSADCase immunoreactivity was present in the presynaptic matrix and around the external surfaces of small synaptic vesicles. Control sections treated with preim-

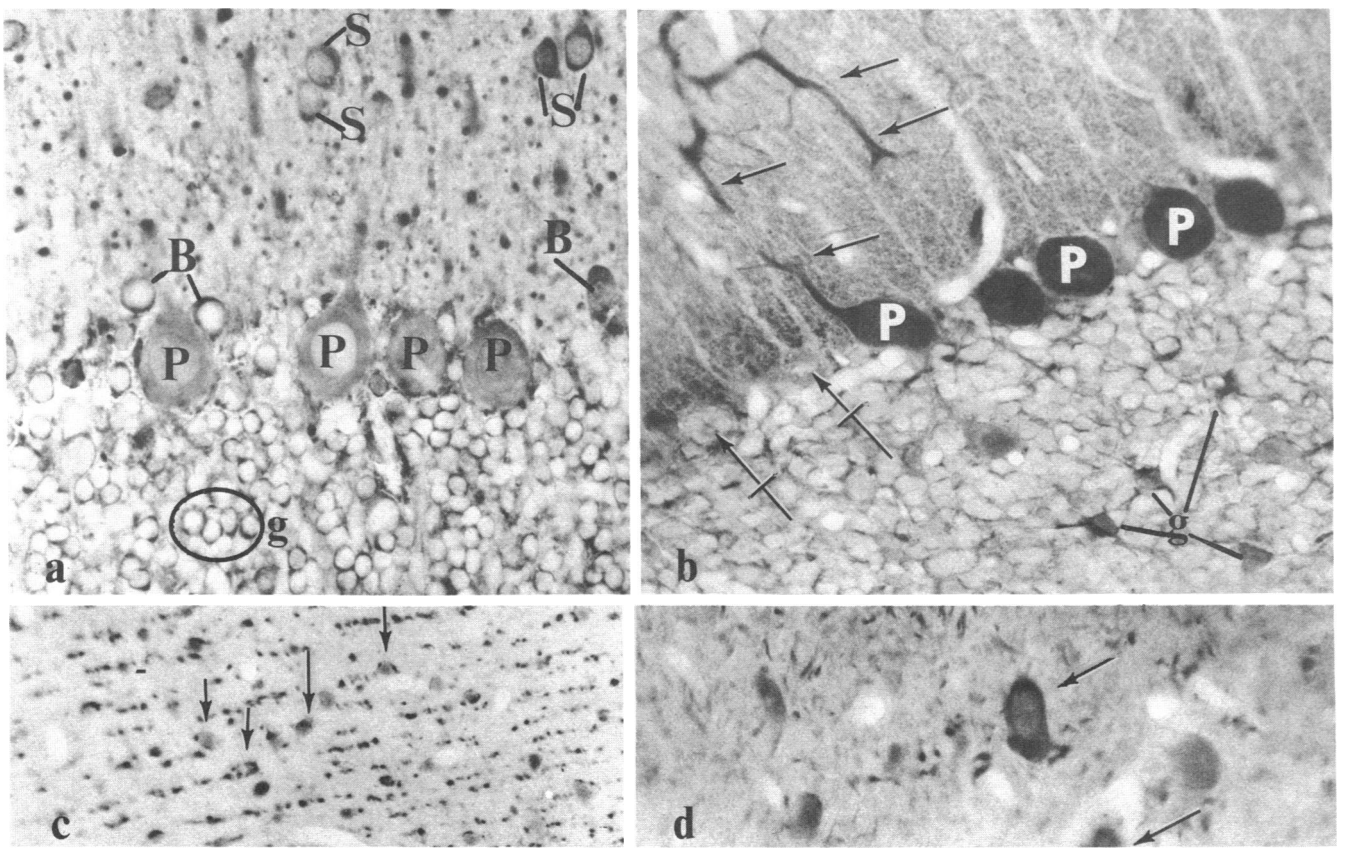

FIG. 2. Rat cerebellar cortex and dentate nucleus labeled by immunoreactivity with anti-CSADCase antibodies and the indirect peroxidase conjugate and PAP method visualized by *p*-dimethylaminoazobenzene. (*a*) Vermis lobule I, sagittal section in the normal rat. Note the staining of four Purkinje cell somata (P) and their primary dendrites, several stellate cells (S) and basket cells (B), and numerous granule cells (g). (*b*) Vermis lobule V, sagittal section in rat pretreated with colchicine. Note the intense staining of several Purkinje cells (P) and their primary dendrites (arrows) and the lack of staining in others (crossed arrows). In the granular layer, a number of granule cells (g) are heavily immunoreactive. (*c*) Vermis lobule VI, horizontal section in normal rat. Note the labeled varicose parallel fibers running in separated ranks from left to right. In between are cross-sectioned Purkinje cell dendritic trunks (arrows). (*d*) Dentate nucleus with several CSADCase immunoreactive neurons (arrows). (×200.)

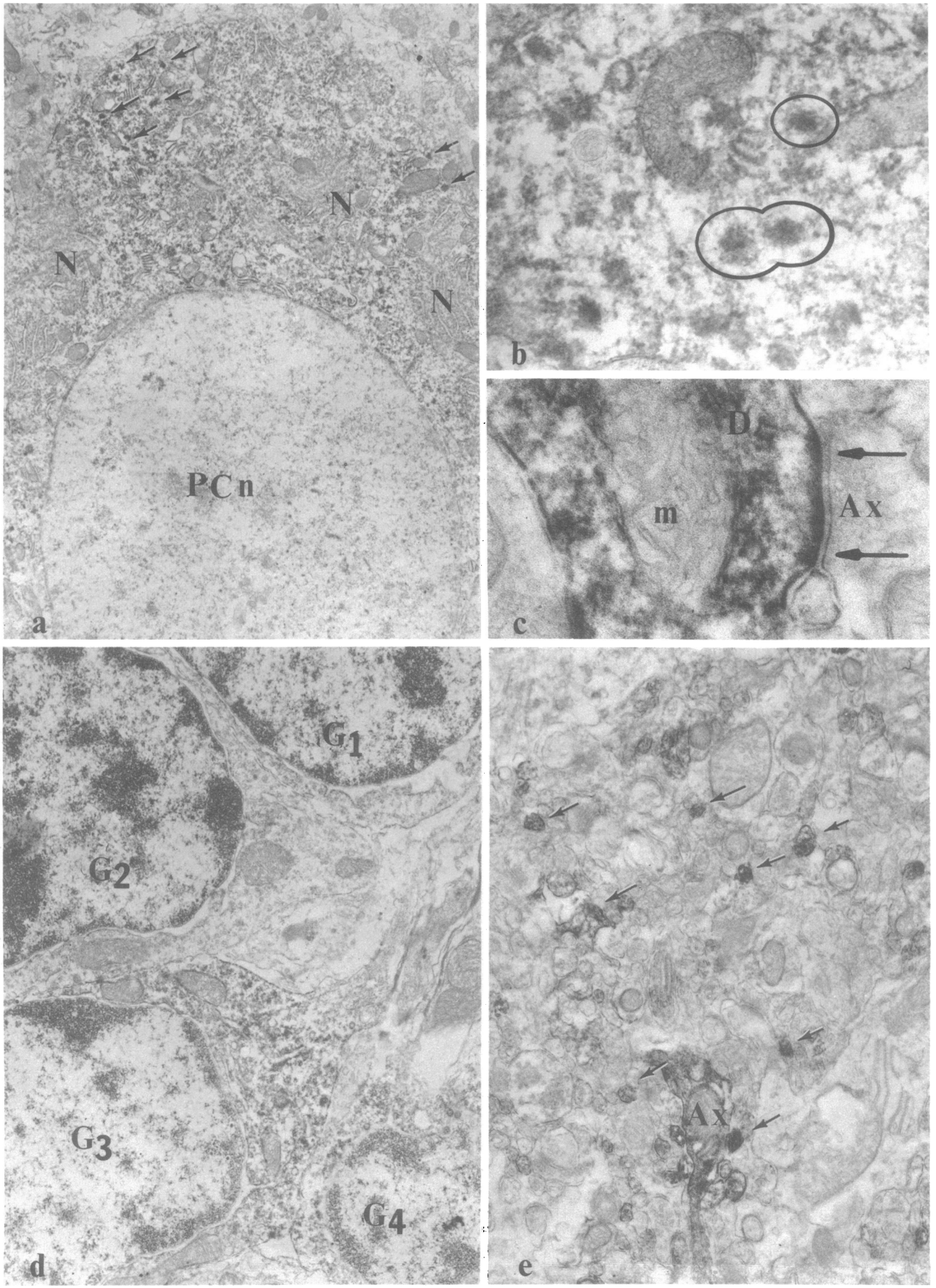

FIG. 3. *(Legend appears at the bottom of the next page.)*

mune serum, anti-ovalbumin antibodies, and anti-CSADCase antiserum preabsorbed with CSADCase showed no immunoreactivity.

DISCUSSION

The match between cerebellar neurons with CSADCase immunoreactivity and those with [^{3}H]taurine uptake systems with respect to cell type and location supports the hypothesis that many Purkinje neurons, stellate, basket, Golgi cells, granule cells, and some deep cerebellar nuclear neurons synthesize and utilize taurine. Taurine has been implicated as an inhibitory transmitter (1, 19) and, therefore, its presence in neurons that are known to have inhibitory roles (20) in the cerebellar circuits, such as Purkinje cells, stellate, basket, and Golgi neurons, might be expected. However, the presence of taurine in granule cells and in deep cerebellar projection neurons, which are generally considered to be excitatory elements (21), calls for a reexamination of their roles in the cerebellar circuits.

The size and locations of taurine-labeled neurons in all of the deep nuclei indicate that they may be projection neurons. [^{35}S]Methionine is readily converted *in vivo* and *in vitro* in rat brain (22, 23) to ^{35}S-labeled cysteine, taurine, and sulfate and is incorporated into brain proteins. Recently the cerebellofugal projections from the dentate nucleus in rhesus monkey and lateral nucleus in rats have been traced in autoradiographic studies after *in vivo* microinjections of high-specific-activity [^{35}S]-methionine directly into the nucleus (14). Neurons in the injection sites rapidly transport ^{35}S-labeled material through their massive axonal plexuses to the rest of the brain, particularly the brain stem and thalamus according to a strict topographic organization. These projections likely belong to the taurinergic cerebellar nuclear neurons. Whether any taurine nuclear neurons are local-circuit neurons remains to be established.

In the Purkinje cell, primary dendrites, their branch points, and the cell body represent the major synthetic and uptake sites for taurine. These dendritic branch points have a highly specialized structure and innervation (13, 24). They are completely draped by climbing fibers, which synapse on thorns, and by numerous basket and stellate cell axons. It has been proposed that, at these locations, the integration between excitatory and inhibitory influences occurs, and the resultant effect may selectively tune in or out whole segments of dendritic tree distal to these branch points (24, 25). It has been reported that calcium-dependent dendritic spikes develop at these branch points (25, 26). We suggest that taurine might be associated with the calcium dendritic spike process.

Finally, it should be noted that Purkinje cells are chemically heterogeneous. They have been known to contain γ-amino butyrate (21, 27) and motilin (28, 29), and some even contain both (28). The present paper demonstrates that many contain taurine.

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1. Curtis, D. R. & Watkins, J. C. (1960) *J. Neurochem.* **6**, 117–141.
2. Curtis, D. R. & Watkins, J. C. (1965) *Pharmacol. Rev.* **17**, 347–391.
3. Haas, H. L. & Hosli, L. (1973) *Brain Res.* **52**, 399–402.
4. Jasper, H. H. & Koyama, I. (1969) *Canad. J. Physiol. Pharmacol.* **47**, 889–905.
5. Davison, A. N. & Kaczmarek, L. K. (1971) *Nature (London)* **234**, 107–108.
6. Hammerstad, J. P., Murray, J. E. & Cutler, R. W. P. (1973) *Brain Res.* **52**, 399–402.
7. Kaczmarek, L. K. & Davison, A. N. (1972) *J. Neurochem.* **19**, 2355–2362.
8. Lahdesmaki, P. & Oja, S. S. (1972) *Exp. Brain Res.* **15**, 430–438.
9. Schmid, R., Sieghardt, W. & Karobath, M. (1975) *J. Neurochem.* **25**, 5–9.
10. Pasantes-Morales, H., Klethi, J., Urban, P. F. & Mandel, P. (1974) *Exp. Brain Res.* **19**, 131–141.
11. McBride, W. J., Nadi, N. S., Neuss, M. & Frederickson, R. C. A. (1977) *Trans. Am. Soc. Neurochem.* **8**, 91.
12. Piha, R. S. & Saukkonen, H. (1966) *Suomen Kemistilehti* **B39**, 112–114.
13. Palay, S. L. & Chan-Palay, V. (1974) *Cerebellar Cortex, Cytology, and Organization* (Springer-Verlag, Berlin).
14. Chan-Palay, V. (1977) *Cerebellar Dentate Nucleus, Organization, Cytology, and Transmitters* (Springer-Verlag, Berlin).
15. Chan-Palay, V. (1981) in *Current Trends in Morphological Techniques*, ed. Johnson, J. E. (CRC Press Inc., Boca Raton, FL), Vol. 2, pp. 53–90.
16. Lin, C.-T., Garbin, J.-Y. & Wu, J.-Y. (1982) *J. Histochem. Cytochem.*, in press.
17. Chan-Palay, V., Wu, J.-Y. & Palay, S. L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2067–2071.
18. Wu, J.-Y., Moss, L. G. & Chen, M.-S. (1979) *Neurochem. Res.* **4**, 201–212.
19. Chan-Palay, V., Ito, M., Tongroach, P., Sakurai, M. & Palay, S. L. (1982) *Proc. Natl. Acad. Sci. USA*, in press.
20. Eccles, J., Ito, M. & Szentágothai, J. (1967) *The Cerebellum as a Neuronal Machine* (Springer-Verlag, Berlin).
21. Wood, J. G., McLaughlin, B. J. & Vaughn, J. E. (1976) in *GABA in Nervous System Function*, eds. Roberts, E., Chase, T. N. & Tower, D. B. (Raven, New York), pp. 133–148.
22. Gaitonde, M. K. & Richter, D. (1956) *Proc. Roy. Soc., London* **145**, 83–99.
23. Gaitonde, M. K. & Richter, D. (1957) in *Metabolism of the Nervous System*, ed. Richter, D. (Pergamon, London), pp. 449–455.
24. Chan-Palay, V. & Palay, S. L. (1970) *Z. Anat. Entwickl-Gesch.* **132**, 191–227.
25. Llinás, R., Freeman, J. A. & Hillman, D. E. (1968) *Science* **160**, 1132–1135.
26. Llinás, R. & Sugimori, M. (1979) in *Development and Chemical Specificity of Neurons, Progress in Brain Research*, eds. Cuénod, M., Kreutzberg, G. W. & Bloom, F. E. (Elsevier, Amsterdam), Vol. 51, pp. 324–334.
27. Obata, K., Ito, M., Ochi, R. & Sato, N. (1967) *Exp. Brain Res.* **4**, 43–57.
28. Chan-Palay, V., Nilaver, G., Palay, S. L., Beinfeld, M. G., Zimmerman, E. A., Wu, J.-Y. & O'Donohue, T. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7787–7791.
29. Nilaver, G., Defendini, R., Zimmerman, E. A., Beinfeld, M. C. & O'Donohue, T. L. (1982) *Nature (London)* **295**, 597–598.

FIG. 3. Electron micrographs of CSADCase immunoreactive neurons and their processes in the rat cerebellum. (*a*) Purkinje cell somata with unlabeled nucleus (PC n) and cytoplasm without immunoreactivity in Nissl substance (N) and mitochondria. The densest foci of immunoreactivity are represented by 120-nm round nonmembrane-associated collections of peroxidase reaction product (arrows, enlarged in Fig. 3*b*). (×5,000.) (*b*) Intensely immunoreactive, 120-nm CSADCase plaques labeled with peroxidase reaction product in somata and particularly in major dendrites of Purkinje cells. (×25,000.) (*c*) Synaptic junction (between arrows) formed by an unidentified axon (Ax) and a CSADCase-immunoreactive dendrite D, containing an unlabeled mitochondrion (m). (×60,000.) (*d*) A field of four granule cells, two of them without staining (G_1, G_2) and two others (G_3, G_4) CSADCase immunoreactive in somata and emerging dendrite. (×9,000.) (*e*) Molecular layer of the cerebellar cortex in sagittal section showing CSADCase-immunoreactive parallel fibers (arrows) (compare with Fig. 2*c*) and an axon of a cortical interneuron (Ax) similarly marked. (×7,500.)